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# Toxicology Studies on Lewisite and Sulfur Mustard Agents: Two-Generation Reproduction Study of Sulfur Mustard (HD) in Rats

## Final Report

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Comprehensive data are not available to evaluate the potential reproductive risk from long-term exposure to sulfur mustard (HD), [bis(2-chloroethyl)sulfide]. Groups of rats (27 females and 20 males/group/ generation) were gavaged with 0, 0.03, 0.1 or 0.4 mg/kg HD for 13 weeks prior to mating, and throughout gestation, parturition and lactation in a 42-week two-generation study. Growth of adult $F_1$ rats of both sexes was reduced by the 0.4 mg/kg exposure. There were no significant effects on reproductive function or pregnancy outcome in either generation. Although not different at birth, growth of the 0.4 mg/kg $F_1$ and $F_2$ offspring was depressed during lactation. A dose-related lesion of the squamous epithelial mucosa of the forestomach was observed in both sexes. The lesion was characterized by thickening of the squamous mucosa with varying degrees of hyperkeratosis. Benign neoplasms of the forestomach were found in about 10% of the intermediate (8/94) and high (10/94) dose groups. The NOEL for toxicity in this study was <0.03 mg/kg and for reproductive effects was >0.4 mg/kg. (SDW)

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I B Sasser 11-30-89
PI Signature Date

## EXECUTIVE SUMMARY

Chemical warfare agents present an obvious risk to individuals suffering acute exposure, but they may also present long-term environmental or occupational health hazards for workers in operations involving these chemical agents. Occupational health standards have not been established for sulfur mustard (HD) [bis(2-chloroethyl)-sulfide] a strong alkylating agent with known mutagenic and suspected carcinogenic properties. Sulfur mustard is used in a number of research laboratories, stored in depot sites throughout the country and occasionally transported to distant sites. The destruction of current stockpiles of HD by the U.S. Army in the near future could create additional environmental and occupational risk. To establish a database for setting environmental and occupational standards, we have conducted studies to evaluate the toxicity, mutagenicity, and reproductive effects of HD using *in vitro* and *in vivo* study systems. The purposes of this multi-generation study were to determine the reproductive consequences and dose-response of continuing chemical exposure of parental males and females and their offspring in a 42-week two-generation study.

Sulfur mustard was administered to three groups of male and female rats ($F_0$) prior to mating, during mating, and after mating until birth of the offspring at which time the male rats were sacrificed. The dams continued to receive sulfur mustard during lactation. At weaning, male and female offspring ($F_1$) of each group were randomly selected to continue on the study; receiving HD during adolescence, mating, and throughout gestation. Again, the parental males were sacrificed at birth of the offspring and the parental females continued to receive HD until weaning of the offspring ($F_2$) at three weeks of age at which time both mother and pups were sacrificed. A fourth group of male and female rats received sesame oil and served as the vehicle controls. Twenty male and 27 female rats were assigned to each of treatment groups and to the vehicle control group for each generation.

Intragastric administration of HD at levels of 0.03, 0.1, and 0.4 mg/kg/day had no adverse effect on reproductive performance, fertility or reproductive organ weights of male and female rats through two consecutive generations. Growth of adult $F_1$ rats of both sexes was significantly reduced ($P < 0.05$) by the 0.4 mg/kg exposure. Although not different at birth,

growth of the 0.4 mg/kg $F_1$ and $F_2$ offspring was depressed at 14 and 21 days of age.

Daily intragastric administration of 0.4 mg/kg of HD to parental rats in the $F_0$ and $F_1$ generation caused no gross or microscopic lesions in testes, epididymis, prostate, seminal vesicles, ovaries, uterus, or vagina. The forestomach was the primary target organ for HD. Benign neoplasms of the forestomach were found in about 10% of the 0.1 mg/kg and 0.4 dose groups. A dose-related lesion of the squamous epithelium of the forestomach was observed at the 0.03 mg/kg dose level although the lesion was mild compared to the other treatment groups. The characteristics of the epithelial lesions were similar to those of a 90-day subchronic study except that in this study, mild lesions were observed in over 50% of the animals receiving 0.03 mg/kg of HD whereas in the subchronic study lesions were limited to the 0.3 mg/kg group except for one animal receiving 0.1 mg/kg.

In conclusion, exposure to HD at levels of .03, 0.1 and 0.4 mg/kg/day did not have any adverse effect on reproductive performance or fertility of male or female rats through two consecutive generations. The No-Observable-Effect-Level in this study was $<0.03$ mg/kg for toxicity and $> 0.4$ mg/kg for reproductive effects.

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## INTRODUCTION

Chemical warfare agents present an obvious risk to individuals suffering acute exposures and may also present certain environmental or occupational health hazards for workers in operations involving these chemical agents. Although considerable information is known concerning the acute effects of these agents, little information is available concerning the long-term hazards of these materials, including reproductive effects. In recent years, the potential for exposure of women of childbearing age has increased as a consequence of changing legal and socioeconomic factors. Because increasing numbers of reproductively competent women are now in the work force, the long term effects of these agents on reproduction must be considered. It is therefore necessary that potentially toxic and mutagenic chemicals be identified, and that a database be established for the development of hazard evaluations and occupational health standards for these chemicals.

The two general categories of vesicants are typified by lewisite [dichloro(2-chlorovinyl)arsine] and sulfur mustard (HD) [bis(2-chloroethyl) sulfide] Cassarett and Doull, 1976. Contact with these chemicals produces severe skin burns. Recently, a renewed interest in these chemicals was generated by the release of a United Nations report that contained substantial evidence that Iraq was manufacturing and using these agents as chemical warfare agents (Marshall, 1984).

The mustard compounds (both sulfur and nitrogen) are biochemically related to a group of cytotoxic alkylating agents, including the ethylenimines, sulfonic esters, epoxides and n-alkyl-n-nitroso compounds (Wheeler, 1962). These chemicals react rapidly with certain functional groups of proteins (OH, $NH_2$, and SH) to alter their metabolic activity. In aqueous solutions, both sulfur and nitrogen mustard hydrolyze to form cyclic sulfonium or immonium forms, respectively, which, in turn, will react with nucleophilic sites. The sulfur mustard reaction proceeds more rapidly to the reaction with nucleophiles than does nitrogen mustard and is independent of the concentration of nucleophiles present (Fox and Scott, 1980). The cytotoxic, mutagenic, and carcinogenic properties of mustard compounds have been studied extensively (Fox and Scott, 1980), but most of this data relate to

nitrogen mustard because sulfur mustard is a more toxic and chemically reactive vesicant.

Relevant chemical and physical properties of sulfur mustard are summarized in Table 1. In aqueous solutions, sulfur mustard rapidly hydrolyzes to form a cyclic sulfonium salt, β-chloroethyl-ethylenesulfonium chloride. This salt reacts with water to form β-chloroethyl-β-hydroxyethyl sulfide and hydrochloric acid. Subsequent hydrolysis of the sulfide, presumably through the intermediation of a second sulfonium salt, forms thiodiglycol (Anslow et al., 1948). These workers have investigated the toxicity of these derivatives of sulfur mustard and a number of other intermediates isolated from hydrolysates of sulfur mustard. They found that two of the derivatives, β-chloroethyl-β-hydroxyethyl sulfide and thiodiglycol, were relatively nontoxic.

Few values are available in the literature for the $LD_{50}$ of sulfur mustard. Table 2 includes $LD_{50}$ data for sulfur mustard administered to mice, rats and rabbits. Haskin (1948) reported that extensive edema occurred at the site of administration of nitrogen mustard (IP and subcutaneous) and that

TABLE 1. Relevant Chemical and Physical Properties of Sulfur Mustard, Bis(2-Chloroethyl)Sulfide[a]

| | |
|---|---|
| CAS #: | 505-60-2 |
| RETCS #: | WQ0900000 |
| Structural formula: | $Cl\text{-}CH_2\text{-}CH_2\text{-}S\text{-}CH_2\text{-}CH_2\text{-}Cl$ |
| Molecular weight: | 159.1 g |
| Density at 25°C: | 1.3 g/ml |
| State: | Colorless, oily liquid |
| Vapor pressure at 20°C: | 0.072 mm |
| Decomposition temperature: | 149-177°C |
| Solubility in water at 25°C: | 0.68 g/L |
| Hydrolysis | |
| Rate ($T_{\frac{1}{2}}$ at 25°C, pH 7): | 8.5 min |
| Products: | Thiodiglycol, chloride |
| Army Abbreviation | HD |

[a]Rosenblatt et al., 1975 and Windholz, 1983.

TABLE 2. $LD_{50}$ Values[a] of Various Routes of Administration for Sulfur and Nitrogen Mustard

| Chemical | Route of Administration[b] | $LD_{50}$ (mg/kg) Rat | Rabbit | House |
|---|---|---|---|---|
| Sulfur Mustard | IV | 0.7, 3.3 | 1.1 | 8.6 |
| | SC | 1.5 | 20 | 20 |
| | Skin | 5 | 92 | 92 |
| Nitrogen Mustard | IV | 1.1 | 1.6 | 2 |
| | SC | --- | 3 | 2.6 |
| | IP | 1.6 | --- | 2.4 |
| | Oral | 10 | 5 | 10 |
| | Skin | 12 | 12 | 29 |

[a]Registry of Toxic Effects of Chemical Substances, D.V. Sweet, 1987; Fox and Scott, 1980.
[b]IV = intravenous; SC = subcutaneous; IP = intraperitoneal.

diarrhea, dyspnea, and anorexia were common observations. Death occured in rats within 3 to 4 days after administration at dose levels of 1.8 to 3.1 mg/kg and within 5 to 19 days of administered doses of 1 to 1.2 mg/kg.

Relatively little is known concerning the effects of HD on development and reproduction. Chronic inhalation exposure of male rats to sulfur mustard (0.1 mg/m³) was reported to produce significant dominant lethal effects, but exposure of pregnant females to the same concentrations for a shorter time interval failed to induce fetal malformations (Rozmiarek et al., 1973). McNamara et al. (1975) subsequently concluded from these same data that there were no differences between the control and experimental groups and no evidence of mutagenesis. It is difficult to resolve the apparent conflict between the conclusions of these two reports, but the fetal mortality values presented in the McNamara report suggest at least a trend for a significant dominant lethal effect. Complete control data are not included in the report and statistical evaluation of the results is not presented, but percentages of fetal death at week 12 were 4.12, 4.24, and 21.05 for controls, 0.001 and 0.1 mg/m³ exposure groups, respectively.

The teratogenic potential of HD was studied in rats exposed to two concentrations of inhaled HD (0.001 and 0.1 mg/m$^3$) during each of the 3 weeks of gestation or throughout the entire gestation period (McNamara et al., 1975). No evidence of dose-related fetal mortality or gross abnormalities was noted. Teratology studies, following the segment II teratology protocol, were recently conducted in rats and rabbits by Hackett et al. (1987). Rats were exposed to 0.5-2.0 mg/kg HD by gastric intubation from 6 to 15 days of gestation (dg) and were sacrificed on dg 20. No evidence of a teratogenic response to HD was observed since fetal effects occurred only at doses exhibiting signs of maternal toxicity. Likewise, fetal development of rabbits exposed to 0.4-0.8 mg/kg HD between 6 and 19 days of gestation was not affected even though maternal mortality was induced at the highest dose. These results suggest that HD is not teratogenic in rats and rabbits since fetal effects were observed only at dose levels that induced frank maternal toxicity.

Comprehensive data are not available to evaluate the potential risk to reproduction from long-term occupational exposure to sulfur mustard. The purposes of this multi-generation study were to determine the reproductive consequences and dose response of continuing chemical exposure of parental males and females and their offspring in a 42 week two-generation study.

## MATERIALS AND METHODS

### SULFUR MUSTARD

#### Procurement and Characterization

The sulfur mustard used in these studies was 2,2',dichlorodiethyl sulfide, also known as Bis(2-choroethyl)sulfide or distilled mustard (HD).

The sulfur mustard was supplied by the U.S. Army Medical Research Institute for Chemical Defense (USAMRICD), Chemical Surety/Safety Office, Aberdeen Proving Ground, Edgewood Arsenal MD from lot No. HD-U-4244-CTF-N-1, previously designated Lot No. ICD-HD-1. The material was prepared August 31, 1981 and analyzed for purity September 4, 1984 by Captain William Beaudry and Linda Szafdraniec (Research Directorate Chemical Research) by nuclear magnetic resonance. Purity, calculated on a weight basis, was 97.3%. There were two impurities with concentrations of 1.2% (assumed to be dithiane) and 1.5% (identity unknown). Material from this lot has been proposed as the standard analytical reference for U.S. Army Medical Research and Development Command (USAMRDC) and USAMRDC has agreed to retain aliquots of this material to comply with the requirements of Good Laboratory Practices (GLP).

A shipment of 25 ml of HD (in two ampules) was delivered on March 7, 1985 by a team from the U.S. Army Technical Escort Unit. The ampules were inspected and found to be intact. Subsequently the HD was transferred from the ampules into 30-ml Wheaton bottles, sealed and stored in secondary unbreakable containers in a refrigerated storage container at approximately 6°C.

#### Selection and Characterization of Diluent

Sulfur mustard is relatively insoluble (680 mg/L) and also is rapidly hydrolyzed in water, therefore sesame oil was employed as the diluent for dosing solutions in this study. This selection was not only based on the chemical and physical properties of the compound, but also on the lack of a toxic response of the vehicle when introduced into the stomach of the animal. Corn oil is commonly the vehicle used for the administration of water-

insoluble compounds; however, Hackett et al. (1987) concluded from data in the literature that corn oil may not be appropriate for reproductive studies because of its high steroid content and recommended using sesame oil in their studies of the teratology of sulfur mustard. Sesame oil contains no preservatives, appears to be stable when stored under proper conditions, is relatively low in steroids and is readily available.

The sesame oil (Hain Pure Food Company, Los Angeles, CA) used in this study was purchased locally in one quart bottles and numbered according to lot and bottle. Peroxide analysis of each lot of sesame oil was performed at the beginning of the study or when purchased and periodically throughout the study to provide a measure of oxidation as an indication of oil rancidity. The method measures the ability of the oil to oxidize aqueous iodide. Only oil in which the peroxide content was less than 10 meq/kg was used in the study.

The results of the peroxide analyses of the sesame oil used are given in Table 3. The amount of peroxide in the sesame oil was well within the acceptable limits of 10 meq/kg.

Preparation of Solutions for Administration

The HD dosing solutions administered to the animals were prepared in advance and stored in a refrigerator at approximately 6°C. The general procedure was to determine in advance the amount of neat HD needed, based on the volumes to be prepared and the final concentrations desired. This volume was then removed from the bottle of neat HD and thoroughly mixed into a known volume of sesame oil. Aliquots of this intermediate concentration were then diluted further to give the final concentration needed for the dosing solutions. Aliquots of the final solutions were placed in Wheaton bottles with teflon-lined sepa and aluminum caps. Each Wheaton bottle contained sufficient volume of HD-sesame oil for 1 day's use. The bottles were labeled with the name and the concentration of the agent (HD) and placed into a secondary unbreakable container which was identified by chemical name, concentration, lot number and date prepared.

TABLE 3. Analyses of Sesame Oil for Peroxide

| Lot No. | Date Purchased | Assay Date | Container Identification | Peroxide meq/kg |
|---|---|---|---|---|
| 11421/B | 5/1/85 | 11/20/85 | G | 5.7 |
| | | 12/17/85 | D | 5.6 |
| 50775-15 | 11/19/85 | 11/21/85 | 1 | 4.8 |
| 50775-29 | 1/15/86 | 1/17/86 | 1 | 6.1 |
| | | 2/19/86 | 7 | 5.7 |
| | | 3/20/86 | 11 | 8.8 |
| 50775-49 | 3/14/86 | 3/20/86 | 2 | 4.1 |
| | | 4/21/86 | 9 | 3.9 |
| | | 5/22/86 | 12 | 4.6 |
| 50775-82 | 6/12/86 | 6/12/86 | 1 | 5.7 |
| | | 6/12/86 | 2 | 5.6 |
| | | 7/11/86 | 2 | 6.5 |
| 50775-95 | 7/8/86 | 8/20/86 | 8 | 6.0 |
| | | 9/16/86 | 11 | 6.0 |

Analytical Procedures

Methods were developed for the assay of HD in sesame oil by gas chromatography, using a capillary column and flame-ionization detection. The assay was complicated by the high boiling points of some components in sesame oil. As a result, the temperature of the capillary-column inlet had to be maintained at 200°C. The procedure consisted of diluting 0.50 ml of the HD-sesame oil sample with 0.50 ml of 18.7 ng/ul 2,4-dichlorotoluene (DCT) in isooctane, contained in a 1.5-ml automatic sampler vial with a Teflon-lined crimped-top cap. The DCT was used as an internal standard for the assay. A Hewlett-Packard 5840A gas chromatograph and 7672 automatic sample changer were used with a DB-5 capillary column (J & W Scientific). The method can detect as low as 0.01 mg/ml.

Results of samples analyzed using this method are presented in Table 4. Theoretical and analyzed values were essentially the same, especially at the higher concentrations. Some deviation between theoretical and analyzed values was seen at the low concentration. This may have resulted from a lack of precision of the method or could be the result of degradation by the sesame oil as the percentage of oil increased. When HD samples were repeatedly analyzed, little evidence of degradation was seen during the storage period in sesame oil.

The S-value in Table 4 represents the relative sensitivity of the gas chromatograph to HD as compared to the internal standard, 2,4-dichlorotoulene (DCT), and can be used to compare samples analyzed at different times. It is defined by the equation:

$$S = (A_{HD}/A_{DCT})/[DCT]/[HD])$$

where the A's designate chromatographic-peak areas of the compounds HD or DCT and the brackets designate concentrations of the compounds in mg/ml. The long-term variation of the HD can be estimated by the constancy of these data over time. The gradual decrease of the S-value with time indicates that the HD concentration of the stock solution tended to decrease slightly throughout the study.

Table 4. Sulfur Mustard Concentrations (ug/ml) of Dosing Solutions Analyzed for the Two-Generation Reproduction Study

| Date Prepared | Date Analyzed | 240* | 180* | 60* | 18* | S-Value** |
|---|---|---|---|---|---|---|
| | | ug/ml | | | | |
| 11-26-85 | 11-26-85 | -- | 180 | 58 | 17 | 0.576 |
| | 12-3-85 | -- | 172 | 58 | 16 | |
| 12-3-85 | 12-3-85 | -- | 180 | 57 | 16 | 0.542 |
| | 12-17-85 | -- | 177 | 60 | 17 | |
| | 1-3-86 | -- | 171 | 55 | 17 | |
| 12-17-85 | 12-17-85 | -- | 180 | 60 | 16 | 0.576 |
| | 1-3-86 | -- | 171 | 54 | 20 | |
| 1-3-86 | 1-3-86 | -- | 180 | 48 | 18 | 0.532 |
| | 1-20-86 | -- | 174 | 71 | 16 | |

Table 4. Continued

| Date Prepared | Date Analyzed | 240* | 180* | 60* | 18* | S-Value** |
|---|---|---|---|---|---|---|
| | | ug/ml | | | | |
| 1-16-86 | 1-16-86 | -- | 180 | 59 | 18 | 0.518 |
| | 2-4-86 | -- | 188 | 62 | 17 | |
| 1-31-86 | 1-31-86 | 240 | -- | 51 | 17 | 0.641 |
| | 2-24-86 | 228 | -- | 51 | 17 | |
| 2-14-86 | 2-24-86 | 240 | -- | 60 | 20 | 0.530 |
| | 2-28-86 | 242 | -- | 62 | 19 | |
| 2-28-86 | 2-28-86 | 240 | -- | 63 | 18 | 0.517 |
| | 3-17-86 | 246 | -- | 61 | 18 | |
| 3-14-86 | 3-14-86 | 240 | -- | 67 | 20 | 0.517 |
| | 3-31-86 | 240 | -- | 52 | 18 | |
| 3-28-86 | 3-28-86 | 240 | -- | 57 | 16 | 0.526 |
| | 4-14-86 | 228 | -- | 56 | 15 | |
| 4-11-86 | 4-11-86 | 240 | -- | 58 | 17 | 0.492 |
| 5-2-86 | 5-23-86 | 240 | -- | 56 | 16 | 0.425 |
| 5-16-86 | 5-23-86 | 240 | -- | 56 | 14 | 0.440 |
| 5-29-86 | 6-4-86 | 240 | -- | 58 | 16 | 0.453 |
| 6-19-86 | 6-20-86 | 240 | -- | 62 | 17 | 0.377 |
| 7-2-86 | 7-3-86 | 240 | -- | 77 | 20 | 0.405 |
| 7-18-86 | 7-21-86 | 240 | -- | 60 | 18 | 0.490 |
| 7-30-86 | 8-4-86 | 240 | -- | 59 | 17 | 0.437 |
| 8-12-86 | 8-19-86 | 240 | -- | 57 | 17 | 0.457 |
| 8-29-86 | 9-8-86 | 240 | -- | 62 | 18 | 0.438 |

* Theoretical or target concentrations for the 0.4, 0.3, 0.1 and 0.033 mg/kg dose levels were 240, 180, 60 and 18 ug/ml, respectively.
**S-value represents the relative sensitivity of the gas chromatograph to HD compared to the internal standard, DCT.

## ANIMAL MAINTENANCE

Four week old male and female rats of Sprague-Dawley derivation were obtained from Charles River Laboratories, Inc., Portage, MI facility and quarantined in isolation for about 3 weeks until a health evaluation was completed. The Sprague-Dawley rat was selected because it has been used in a number of previous reproductive studies at PNL including gavage studies of sulfur mustard thereby providing information for dose estimation. During quarantine the rats were group housed, separated by sex, in stainless-steel wire bottom cages placed on automatic flush racks with an automatic watering system.

The environmental conditions specified for the animal rooms were temperatures of 72 ± 3°F, relative humidity of 50 ± 15%, and a lighting cycle of 12 hours on and 12 hours off. Certified Rodent Chow (#5002) was purchased from Purina and drinking water was provided *ad libitum*. Drinking water supplied to the animal rooms was passed through a reverse-osmotic purification unit containing two particle filters and a carbon filter.

Near the end of quarantine 11 rats were subjected to a health evaluation and tested for antibodies to viral pathogens. No significant pathogens or lesions were found.

Following isolation the rats were weighed and assigned to the appropriate treatment groups by sex and weight by means of a formal randomization statistical package (see Statistical Methods). Each animal was assigned an individual identification number by means of a metal ear tag. The animals were individually housed in wire bottom cages on flush racks and cage cards were used to indicate the animal number and treatment group. Prior to parturition (no later than dg 17) and during lactation the females were housed in solid bottom littering cages (1 litter per cage) utilizing hardwood chip bedding.

### Experimental Design

The experimental design for the two-generation reproduction study is outlined in Figure 1. HD was administered to three groups of 8 week old male and female rats prior to mating, during mating, and after mating until birth

of the offspring at which time the male rats were sacrificed. The dams continued to receive HD during lactation. At weaning, male and female offspring ($F_1$) of each group were randomly selected to continue on the study; receiving HD during adolescence, mating, and throughout gestation. Again, the parental males were sacrificed at birth of the offspring and the parental females continued to receive HD until weaning of the offspring at 3 weeks of age. A fourth group of male and female rats received sesame oil and served as the vehicle controls. Twenty male and 27 female rats were assigned to each of three treatment groups and to the vehicle control group for each generation (Table 5).

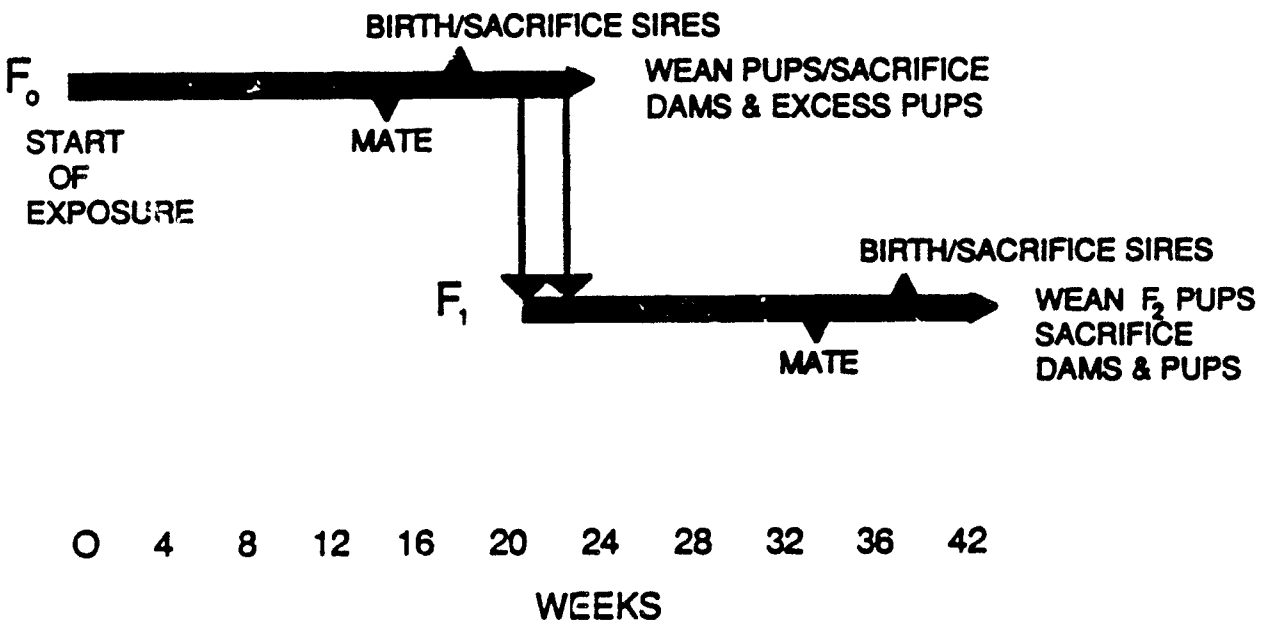


Figure 1. Experiment design.

TABLE 5. Treatment groups of the HD Two-Generation Reproduction Study

| | Number of Males | Number of Females | Number of Dose Levels | Total |
|---|---|---|---|---|
| $F_0$ Generation | | | | |
| HD Exposure Groups | 20 | 27 | 3 | 141 |
| Vehicle Control | 20 | 27 | 1 | 47 |
| Health Screen | -- | -- | - | 11 |
| $F_1$ Generation | | | | |
| HD Exposure Groups | 20 | 27 | 3 | 141 |
| Vehicle Control | 20 | 27 | 1 | 47 |

Administration of Sulfur Mustard

Solutions of the appropriate concentration of sulfur mustard in sesame oil were administered to the animals by intragastric intubation, 5 days per week for 13 weeks, until the beginning of the mating period. During gestation, pregnant female rats were dosed 7 days per week. Except for pregnant rats, animals were not dosed on holidays unless a minimum of 4 doses per week could not otherwise be achieved. Dose levels were calculated weekly based on the animal weight, except during pregnancy when the dose was based on the body weight at day 0 or 6 of gestation. Doses were administered in a constant volume of 1.67 ml/kg of body weight. Vehicle control animals were given an appropriate volume of sesame oil.

Dose levels selected for this study were based on data obtained from a dose-range study in pregnant rats, a 3-week rat teratology study and a 90-day subchronic study in male and female rats (Hackett et al., 1987). When doses of 0, 5, 1.0 and 2.0 mg/kg weight were given to pregnant rats for 10 days, beginning on day 6 of gestation, extragestation weight gain was reduced at all doses in a dose-related fashion, but no evidence of teratogenicity was observed. In the 90-day subchronic study, a dose of 0.3 mg/kg significantly reduced weight gain in both sexes compared to controls and produced lesions

in the forestomach. Since it was desired to select doses such that the highest dose induced toxicity but not mortality in the $F_0$ animals, the low dose not produced any evidence of toxicity, and intermediate dose produce minimal observable toxic effects, dose levels were set at 0.3, 0.1 and 0.03 mg/kg. No observable effects were apparent after 10 weeks of exposure, therefore the 0.3 mg/kg dose level was increased to 0.4 mg/kg in order to demonstrate signs of toxicity in the highest dose group. To avoid confusion, this group will be referred to as the 0.4 mg/kg group throughout this report.

Mating Procedures

Breeding of the $F_0$ and $F_1$ adult females commenced after each generation had been gavaged with HD for 13 weeks. Females were randomly matched with a male rat of the same dose group for one week; those females which did not mate during the first breeding week were reassigned to a second male and cohabited a second week. During the 14-day breeding period each female was transferred to the male cage in the late afternoon and was removed each morning and examined for the presence of sperm plugs or sperm in vaginal smears; the morning on which sperm were found was designated as day 0 of gestation. Females becoming pregnant during the 14-day breeding period were selected for continuation in the study. Females not mating during this period were caged with a male which had been proven fertile from the previous breedings, to insure sufficient number of pregnant animals for continuation of the study in the event of reproductive failures in females of the 14-day breeding groups. These females were then necropsied after the study animals had been selected. For $F_1$ matings, cohabitation of siblings was avoided.

Procedures for Newborn Pups

Pregnant females were checked twice each day beginning on day 17 of gestation. At birth the litters were weighed; pups were counted, sexed and examined for viability and gross abnormalities. The date of parturition was recorded and appearance and behavior of dams and pups were observed daily. On day 4 after delivery, the offspring were weighed and the litters were standardized to four male and four female pups per litter by random

selection; excess pups were killed. If it were not possible to maintain an equal sex distribution within the litter because of a disproportionate sex distribution, a partial adjustment was made in order to maintain a litter size of 8. Litters of less than 8 were not adjusted. Each pup of the litter was uniquely identified with markings on the paws with India ink. The pups were weighed again at day 14 and 21 of age. Pups were weaned on day 21 of age and male and female pups of the $F_1$ generation were randomly selected from each litter for continuation in the study; the excess pups were killed. All $F_0$ and $F_1$ adult females and the $F_2$ pups were killed at weaning.

Twenty male and 27 female pups within each treatment group were randomly selected from the $F_0$ offspring for the $F_1$ study. Each $F_0$ litter was represented by at least one male and one female unless there was a void of either sex within a litter.

Necropsy and Histological Evaluations

A complete gross necropsy was performed on all rats found dead or in moribund condition and those killed at the scheduled sacrifice. Live animals were fasted overnight, euthanized with 70% $CO_2$ within one day of the last two consecutive dosings with HD and immediately necropsied. Weights of the testis, prostate, epididymis, ovary and uterus were recorded. The lungs were fixed by inserting a blunted needle into the laryngeal lumen through which the fixative was infused. The major organs were stored in 10% neutral buffered formalin (NBF) except for the testes which were fixed Bouin's solution and subsequently washed in 70% ethyl alcohol.

Histopathological evaluations were performed on reproductive organs of the high dose group and control group of the $F_0$ and $F_1$ adults. Tissues evaluated included vagina, uterus, ovaries, testes, seminal vesicles, prostate and epididymides. Histopathologic evaluation of the forestomach (the only target organ identified) was performed at all dose levels.

## STATISTICAL METHODS

The computer software program (DRANDBLK) for randomizing animals into experimental groups is based on a single blocking factor for animal weight. Animal weights for a given study were ordered from lightest to heaviest; blocks of animal weights were then randomly assigned to the treatment groups and the control group. Block sizes were governed by the number of test groups.

Analysis of variance was used to analyze body weight organ weights and forestomach lesion data (SAS, 1985). When the results of the analyses were significant, Tukey's Studentized Range Test was used to delineate intergroup differences among means (Tukey, 1953). A comparisonwise error rate was set at 0.05 for Tukey's Test. Kramer's option was used to analyze unequal data sets (Kramer, 1957). An orthogonal contrast was used to test for a trend in the results repeated over time on the same animal, a randomization test was used to test for differences among growth curves (Zerbe, 1979). This test is a non-parametric statistical test that is based on the absolute area between growth curves and allows for correlation of body-weight measurements over time.

Pairwise comparison of binary response variables between groups was done by chi-square test using the P4F program in the BMDP statistical software (Dixon et al., 1983).

## RESULTS

There were no treatment-related deaths during the study although one $F_0$ and four $F_1$ animals did not survive to scheduled sacrifice. None of these deaths were attributed to chemical exposure. Dosing errors were the probable cause of death in the case of one $F_0$ male (0.1 mg/kg) and two $F_1$ animals (control female and 0.03 mg/kg male). Two $F_1$ male deaths (0.03 and 0.4 mg/kg) were attributed to prolonged problems with incisors.

Body weights of the $F_0$ and $F_1$ adult rats during the pre-breeding periods are presented in Tables 6 and 7. The growth curves of the $F_0$ exposed males and female rats were not significantly different from control values, although the growth rate of the high dose males tended to decline after several weeks of exposure and prior to breeding at 14 weeks (Figure 2). On the other hand, growth rates of the high dose $F_1$ male and female rats selected to continue in the study were significantly reduced ($P < 0.5$) compared with controls beginning 1 or 2 weeks after initiation of gavaging (Figure 3). This difference between $F_0$ and $F_1$ animals is most likely due to the change in dose from 0.3 to 0.4 mg/kg for this group 10 weeks into the study. Again the weight depression appeared to be more severe in the male rats. No significant dose-response was observed for body weight at the lower doses.

Breeding performance during the 2-week lavaging period was not adversely affected by exposure to HD for either $F_0$ or $F_1$ animals (Table 8). Except for the control $F_1$ group and the $F_0$ 0.1 mg/kg group, the female fertility and mating indexes were greater than 70%. Only one female ($F_0$ control) did not deliver live pups, although one $F_1$ female of the 0.1 mg/kg group delivered only one live pup. Male fertility index was at least 80% in both $F_0$ and $F_1$ controls and was not adversely affected by HD exposure.

The only statistically significant ($P < 0.5$) parameter relating to the new born pups was an increase in the sex ratio of the $F_0$ offspring of the high exposure group (Table 9). This is probably not biologically significant. Although not significantly different, litter weights and the number of pups per litter tended to decrease in both $F_1$ and $F_2$ at the high exposure level (Table 9). No significant change was observed for mean live pup weight, number of stillborn offspring or number of grossly abnormal pups.

Table 6. Body Weights (g) of $F_0$ Male and Female Rats Exposed to Sulfur Mustard (Mean ± SE).[a]

| Week | Control | 0.03 mg/kg | 0.1 mg/kg | 0.4 mg/kg |
|---|---|---|---|---|
| | | MALES | | |
| 0 | 317.4 ± 8.1 | 313.4 ± 5.5 | 315.1 ± 6.7 | 310.2 ± 4.5 |
| 1 | 358.6 ± 9.2 | 352.0 ± 7.0 | 356.0 ± 7.8 | 346.1 ± 4.9 |
| 2 | 391.1 ± 11.0 | 385.1 ± 8.1 | 389.3 ± 8.8 | 365.5 ± 5.9 |
| 3 | 419.9 ± 11.6 | 407.4 ± 9.0 | 422.8 ± 9.7 | 400.9 ± 6.9 |
| 4 | 441.7 ± 12.2 | 442.8 ± 9.9 | 452.3 ± 10.8 | 428.1 ± 8.1 |
| 5 | 466.6 ± 14.0 | 464.3 ± 11.0 | 473.8 ± 11.8 | 451.3 ± 8.2 |
| 6 | 485.5 ± 15.0 | 488.8 ± 12.0 | 500.9 ± 13.3 | 473.5 ± 8.6 |
| 7 | 506.3 ± 15.8 | 509.9 ± 12.8 | 524.7 ± 14.9[b] | 491.6 ± 9.4 |
| 8 | 523.3 ± 16.4 | 531.8 ± 12.9 | 539.0 ± 15.4[b] | 504.8 ± 10.1 |
| 9 | 544.7 ± 16.9 | 549.0 ± 13.6 | 557.6 ± 16.2[b] | 518.7 ± 9.5 |
| 10 | 557.5 ± 17.0 | 565.8 ± 14.2 | 575.0 ± 17.5[b] | 532.3 ± 9.4 |
| 11 | 573.0 ± 17.6 | 581.4 ± 14.7 | 587.8 ± 17.6[b] | 540.3 ± 11.2 |
| 12 | 585.7 ± 17.9 | 596.8 ± 15.4 | 602.0 ± 18.5[b] | 547.4 ± 10.1 |
| 13 | 602.2 ± 18.6 | 613.6 ± 16.2 | 614.1 ± 20.4[b] | 555.8 ± 10.5 |
| | | FEMALES | | |
| 0 | 205.2 ± 3.1 | 206.5 ± 2.9 | 206.1 ± 2.4 | 204.3 ± 3.8 |
| 1 | 220.1 ± 3.3 | 223.8 ± 3.2 | 223.6 ± 2.5 | 217.7 ± 3.9 |
| 2 | 236.3 ± 3.8 | 239.5 ± 3.8 | 239.8 ± 2.9 | 228.4 ± 4.3 |
| 3 | 250.0 ± 4.3 | 253.8 ± 4.3 | 251.7 ± 3.0 | 243.7 ± 4.8 |
| 4 | 260.6 ± 4.5 | 263.4 ± 3.9 | 264.3 ± 3.8 | 255.1 ± 5.2 |
| 5 | 265.5 ± 4.6 | 274.5 ± 4.6 | 274.6 ± 4.0 | 262.4 ± 5.0 |
| 6 | 277.5 ± 4.9 | 287.1 ± 5.4 | 282.3 ± 3.9 | 272.9 ± 5.0 |
| 7 | 284.6 ± 4.8 | 296.3 ± 6.3 | 290.6 ± 3.8 | 280.9 ± 5.1 |
| 8 | 288.9 ± 5.3 | 295.1 ± 5.6 | 295.2 ± 4.2 | 283.9 ± 5.9 |
| 9 | 291.8 ± 4.6 | 304.3 ± 5.9 | 301.6 ± 4.8 | 288.5 ± 5.5 |
| 10 | 300.7 ± 5.1 | 309.4 ± 6.2 | 306.9 ± 4.4 | 293.2 ± 5.3 |
| 11 | 302.7 ± 5.2 | 311.6 ± 5.9 | 311.6 ± 4.4 | 296.6 ± 5.0 |
| 12 | 307.1 ± 5.2 | 320.3 ± 6.3 | 318.6 ± 5.1 | 301.3 ± 5.3 |
| 13 | 312.5 ± 5.0 | 328.2 ± 7.2 | 326.9 ± 5.5 | 305.2 ± 5.6 |

[a]N= 20 males and 27 females except as noted. [b]N=19.

TABLE 7. Body Weights (g) of $F_1$ Male and Female Rats Exposed Orally to Sulfur Mustard (Mean ± SE).[a]

| Week | Control | 0.03 mg/kg | 0.1 mg/kg | 0.4 mg/kg |
|---|---|---|---|---|
| | | MALES | | |
| 0 | 56.9 ± 2.7 | 61.3 ± 2.3 | 54.3 ± 1.4 | 49.1 ± 3.5 |
| 1 | 69.6 ± 3.4 | 74.6 ± 3.1 | 70.8 ± 2.3 | 61.3 ± 2.4 |
| 2 | 113.8 ± 5.7 | 117.6 ± 5.3 | 112.9 ± 3.9 | 90.7 ± 4.4* |
| 3 | 176.3 ± 7.5 | 178.5 ± 7.2 | 174.6 ± 5.1 | 139.2 ± 6.5* |
| 4 | 239.9 ± 7.6 | 242.4 ± 10.1 | 233.8 ± 6.2 | 189.8 ± 8.4* |
| 5 | 301.5 ± 8.0 | 301.4 ± 9.4 | 294.2 ± 6.8 | 241.5 ± 10.4* |
| 6 | 359.3 ± 8.0 | 362.0 ± 10.1 | 350.0 ± 7.0 | 287.3 ± 12.5* |
| 7 | 413.5 ± 8.4 | 411.9 ± 11.0 | 397.7 ± 6.4 | 331.9 ± 13.5* |
| 8 | 443.8 ± 8.7 | 445.7 ± 12.1 | 428.8 ± 6.7 | 357.8 ± 14.3* |
| 9 | 476.2 ± 9.6 | 479.0 ± 12.7 | 459.7 ± 7.1 | 383.0 ± 16.1* |
| 10 | 508.3 ± 10.1 | 504.6 ± 14.8 | 484.0 ± 6.9 | 404.1 ± 16.8* |
| 11 | 531.8 ± 10.6 | 527.9 ± 18.5 | 506.8 ± 7.3 | 424.0 ± 17.1* |
| 12 | 556.6 ± 11.5 | 554.1 ± 18.7 | 527.1 ± 7.8 | 442.6 ± 17.3* |
| 13 | 578.7 ± 11.7 | 575.7 ± 20.0 | 548.7 ± 7.8 | 462.0 ± 17.8* |
| 14 | 594.7 ± 12.5 | 609.4 ± 15.9[c] | 565.2 ± 8.8 | 469.9 ± 18.8* |
| | | FEMALES | | |
| 0 | 54.0 ± 1.9 | 58.2 ± 1.8 | 52.8 ± 0.9 | 49.5 ± 2.1 |
| 1 | 67.9 ± 2.0 | 71.8 ± 2.4 | 66.7 ± 1.7 | 58.5 ± 2.0* |
| 2 | 107.6 ± 2.8 | 109.8 ± 3.6 | 99.3 ± 2.5 | 81.9 ± 2.9* |
| 3 | 153.1 ± 3.0 | 152.6 ± 4.1 | 146.5 ± 3.0 | 118.8 ± 4.2* |
| 4 | 188.1 ± 3.1 | 180.3 ± 4.3 | 176.1 ± 3.7 | 151.2 ± 4.7* |
| 5 | 209.3 ± 3.6 | 206.1 ± 5.0 | 201.4 ± 4.0 | 175.6 ± 4.8* |
| 6 | 236.6 ± 4.2 | 229.9 ± 5.3 | 227.4 ± 4.6 | 197.7 ± 5.3* |
| 7 | 257.4 ± 4.9 | 251.0 ± 5.3 | 250.8 ± 4.9 | 218.1 ± 5.5* |
| 8 | 270.7 ± 5.3 | 264.2 ± 5.5 | 260.3 ± 5.3 | 228.0 ± 5.5* |
| 9 | 282.3 ± 4.9 | 277.4 ± 5.6 | 274.1 ± 5.2 | 241.6 ± 5.8* |
| 10 | 296.0 ± 5.6[b] | 290.5 ± 6.3 | 287.8 ± 5.3 | 252.3 ± 6.1* |
| 11 | 304.3 ± 6.4[b] | 299.2 ± 6.1 | 294.7 ± 6.1 | 258.8 ± 6.0* |
| 12 | 316.0 ± 6.4[b] | 310.1 ± 6.4 | 303.4 ± 6.0 | 262.0 ± 6.5* |
| 13 | 322.2 ± 6.8[b] | 315.8 ± 6.6 | 309.4 ± 6.1 | 273.6 ± 6.3* |
| 14 | 333.8 ± 7.1[b] | 326.7 ± 6.9 | 319.8 ± 6.6 | 282.3 ± 7.0* |

[a]N=20 males and 27 females except as noted. [b]N=27. [c]N=19.
*Significantly different from control value by Tukey's Test ($P<0.05$), for each pairwise comparsion.

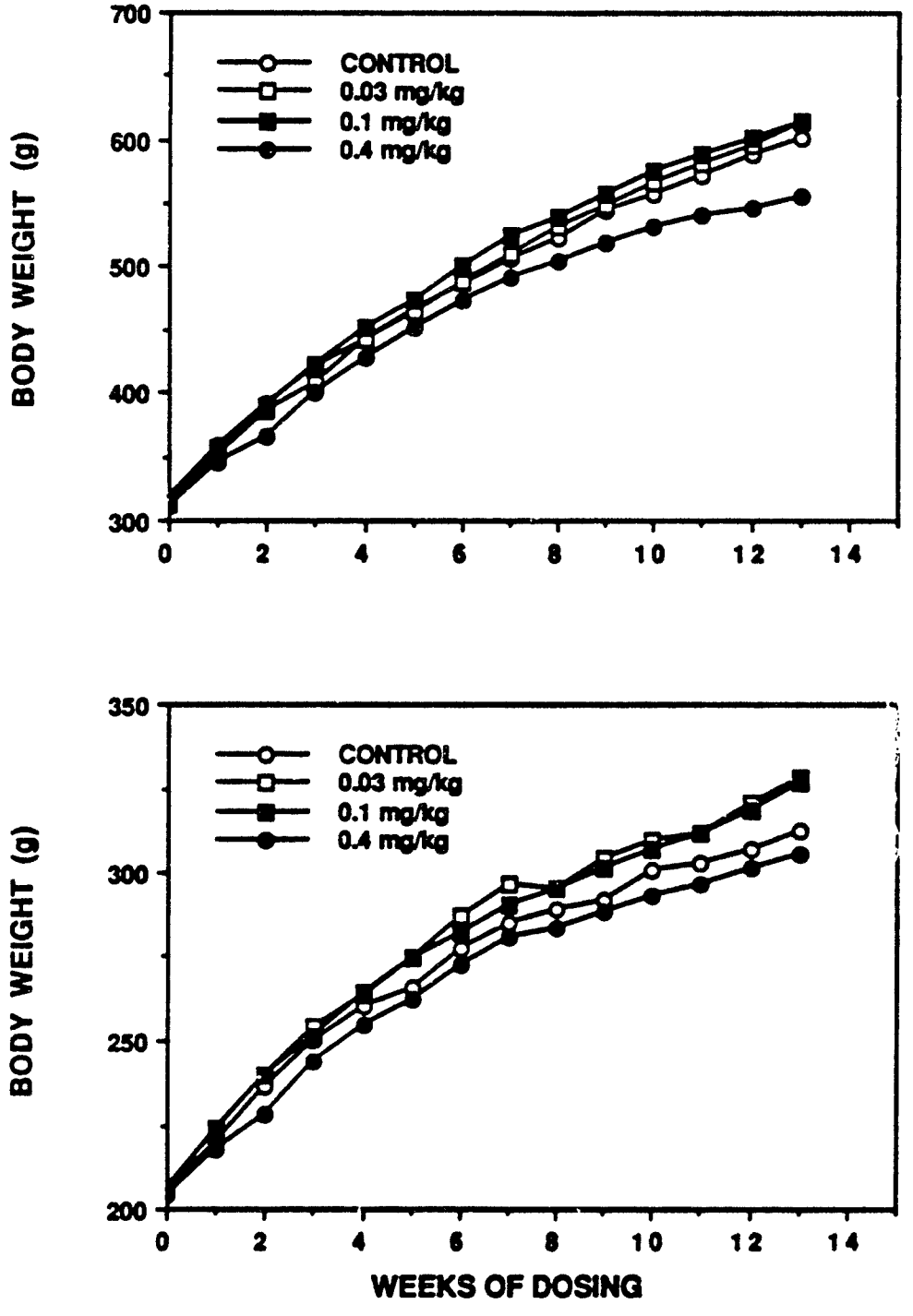


Figure 2. Body weight of $F_0$ male (upper graph) and female (lower graph) rats exposed to HD for 13 weeks.

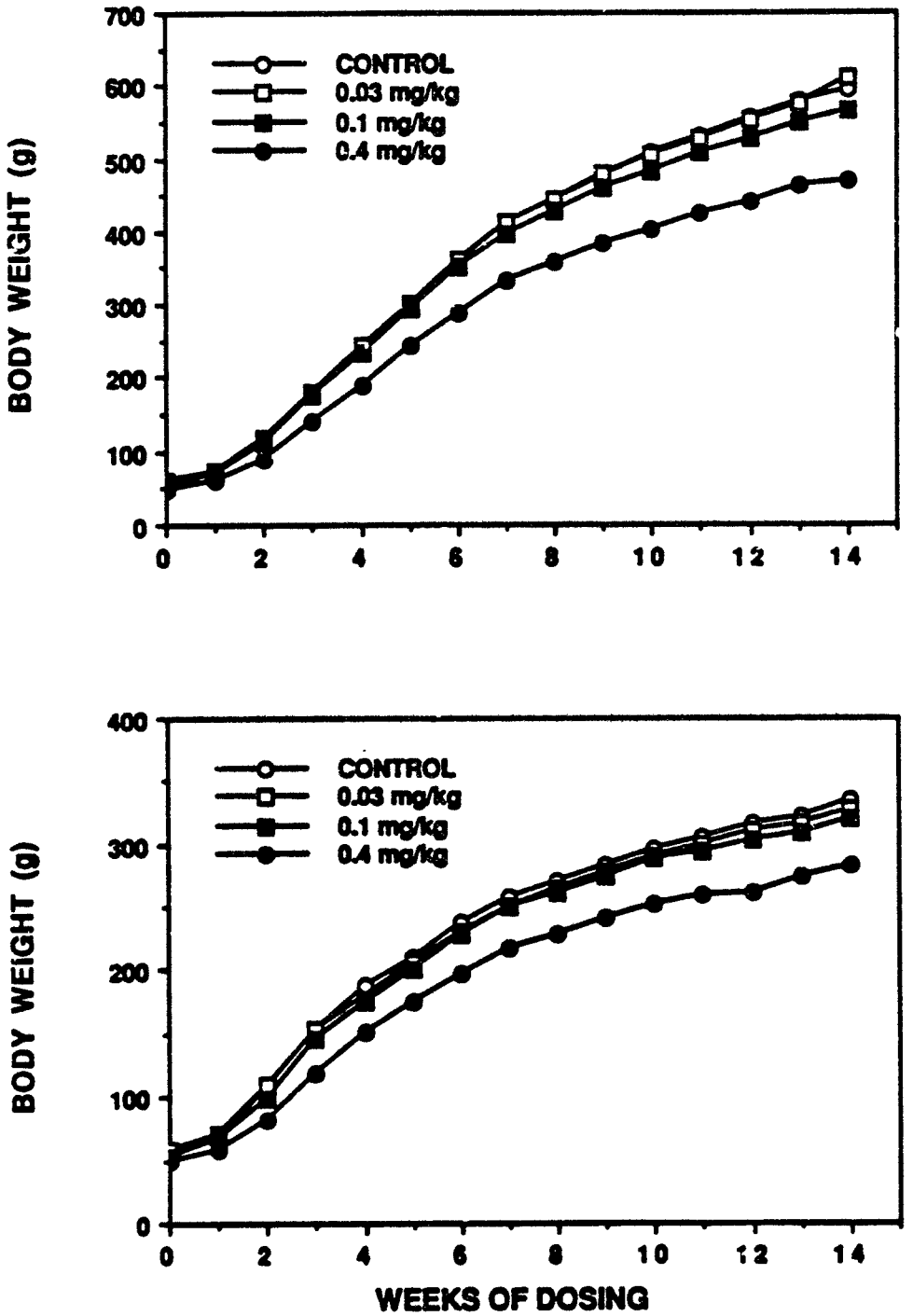


Figure 3. Body weight of $F_1$ male (upper graph) and female (lower graph) rats gavaged with HD for 13 weeks after weaning.

Table 8. Reproductive Performance of $F_0$ and $F_1$-Generation Rats Exposed Orally to Sulfur Mustard.

| HD | Treatment (mg/kg) | | | |
|---|---|---|---|---|
| | 0 | 0.03 | 0.1 | 0.4 |
| | | $F_0$ | | |
| No. Females | 27 | 27 | 27 | 27 |
| No. Matings Detected[a] | 25 | 26 | 23 | 25 |
| No. Pregnant | 19 | 21 | 18 | 21 |
| Females Delivering Live Pups (%) | 95 | 100 | 100 | 100 |
| Fertility Index (%) | | | | |
| Female[b] | 70.4 | 77.8 | 66.7 | 77.8 |
| Male | 80.0 | 80.0 | 80.0 | 85.0 |
| Mating Index[c] | 72.0 | 80.8 | 78.3 | 84.0 |
| | | $F_1$ | | |
| No. Females | 27 | 27 | 27 | 27 |
| No. Matings Detected[a] | 22 | 27 | 25 | 25 |
| No. Pregnant | 15 | 20 | 21 | 24 |
| Females Delivering Live Pups (%) | 100 | 100 | 100 | 100 |
| Fertility Index (%) | | | | |
| Female[b] | 55.6 | 74.1 | 77.8 | 88.9 |
| Male | 80.0 | 95.0 | 90.0 | 85.0 |
| Mating Index[c] | 68.2 | 74.1 | 84.0 | 96.0 |

[a]Number of females in which mating was detected during the 14-day breeding period.

[b]Number of females delivering a litter expressed as a percentage of females placed with a male.

[c]The number of females delivering live litters expressed as a percentage of the females in which matings were detected.

Survival through weaning was unaffected by parental exposure to HD, although a slight non-significant decrease in 0 to 4-day survival was observed at intermediate and high exposures for offspring of the $F_0$ generation.

Although pup weights were not different among exposure groups at birth, growth was significantly depressed at 14 and 21 days of age for $F_1$ and $F_2$ pups of the high exposure group (Table 10). These results suggest that milk production may have been decreased as a result of maternal toxicity to HD during lactation.

Body weights and weights of selected reproductive organs of $F_0$ and $F_1$ males and of $F_0$ and $F_1$ females surviving to the scheduled necropsy are presented in Tables 11 and 12, respectively. Excluded from these results are data of animals classified as early deaths and of non-gravid females not continued in the reproduction phase of the study. Body weights of the high exposure groups of both sexes in both generations were significantly ($P < 0.05$) reduced compared to control animals. Generally, neither absolute nor relative reproductive organ weights of either generation were affected by HD exposure. One exception to this was a significant ($P < 0.05$) decrease in ovary weight of the $F_0$ females in the 0.4 mg/kg dose group; this effect was not observed for the $F_1$ females.

A complete necropsy was performed on all parental animals of each generation and selected tissues were histologically examined. A variety of non-neoplastic lesions in numerous organs and tissues, including those of the reproductive tract, were observed in both control and treated rats but were considered to be incidental in nature and not due to the administration of HD. Non-neoplastic changes in the female genital tract were mainly associated with pregnancy or parturition. The only non-neoplastic lesion observed in the treated animals attributable to the administration of HD was diagnosed as acanthosis of the squamous epithelial mucosa of the forestomach (Table 13). The relative severity and incidence of this lesion is presented in Figure 4 as a function of dose. As can be seen the severity of the lesion was dose-related ($P < 0.05$) and the incidence was approximately the same in each sex of a given treatment group. This lesion was characterized by thickening of the squamous mucosa usually in conjunction with varying degrees of hyperkeratosis. Associated inflammation involving the mucosa and/or submucosa was an infrequent finding.

Table 9. Birth Measurements of $F_0$ and $F_1$-Generation Females Exposed Orally to Sulfur Mustard (Mean ± SE).

| HD | 0 | 0.03 (mg/kg) | 0.1 | 0.4 |
|---|---|---|---|---|
| | | $F_0$ | | |
| No. Litters | 19 | 21 | 18 | 21 |
| Litter Wt.(g) | 81.8 ±4.4 | 87.0 ±4.7 | 82.9 ±7.1 | 68.1 ±6.7 |
| Sex Ratio (Fraction of Males) | 0.44 ±0.027 | 0.53 ±0.021 | 0.51 ±0.048 | 0.58* ±0.033 |
| Live Pup Wt. (g/litter) | 6.28 ±0.09 | 6.47 ±0.12 | 6.38 ±0.19 | 6.11 ±0.18 |
| No. Live Pups | 12.4 ±1.00 | 13.6 ±0.79 | 12.6 ±1.22 | 11.1 ±0.98 |
| No. Stillbirths per Litter | 0.32 ±0.11 | 0.14 ±0.07 | 1.10 ±0.81 | 0.87 ±0.46 |
| No. Abnormal Pups | 2 | 0 | 0 | 1 |
| Pup Survival Index(%) | | | | |
| 0 to 4-day | 98.9 ±0.62 | 98.5 ±0.74 | 90.6 ±6.0 | 93.5 ±4.8 |
| 4 to 21-day** | 100 | 100 | 100 | 100 |
| | | $F_1$ | | |
| No. Litters | 15 | 20 | 21 | 24 |
| Litter Wt. (g) | 76.7 ±6.1 | 84.9 ±5.2 | 85.5 ±4.4 | 65.8 ±4.8 |
| Sex Ratio (Fraction of males) | 0.54 ±0.035 | 0.47 ±0.036 | 0.52 ±0.033 | 0.43 ±0.037 |
| Live Pup Wt. (g/litter) | 6.45 ±0.14 | 6.41 ±0.10 | 6.20 ±0.11 | 6.35 ±0.11 |
| No. Live Pups per Litter | 12.1 ±1.07 | 13.2 ±0.84 | 13.8 ±0.71 | 10.6 ±0.85 |
| No. Stillbirths per Litter | 0.24 ±0.14 | 0.80 ±0.50 | 0.52 ±0.29 | 0.23 ±0.08 |
| No. Abnormal Pups | 0 | 1 | 0 | 0 |
| Pup Survival Index(%) | | | | |
| 0 to 4-day | 99.0 ±0.69 | 92.5 ±4.9 | 94.3 ±4.8 | 98.1 ±0.97 |
| 4 to 21-day** | 98.3 ±1.1 | 100 | 99.4 ±0.62 | 98.4 ±1.6 |

*Significantly different from control value by Tukey's Test ($P<0.05$), for each pairwise comparison.
**Survival indices were determined after reducing the number of pups per litter to 8 on day 4.

Table 10. Growth of $F_1$ and $F_2$ Male and Female Pups During Nursing.

| HD | 0 | 0.03 | 0.1 | 0.4 |
|---|---|---|---|---|
| | | (mg/kg) | | |
| | | $F_1$ | | |
| MALES | | | | |
| Day 4 | 10.6±0.22 | 11.3±0.20 | 10.2±0.19 | 10.2±0.18 |
| Day 14 | 34.6±0.56 | 36.2±0.45 | 34.2±0.39 | 31.2±0.61* |
| Day 21 | 56.9±0.84 | 59.5±1.02 | 55.9±0.80 | 51.8±1.09* |
| FEMALES | | | | |
| Day 4 | 10.1±0.20 | 10.7±0.18 | 9.6±0.19 | 9.8±0.17 |
| Day 14 | 33.7±0.48 | 34.8±0.44 | 32.5±0.40 | 30.7±0.53* |
| Day 21 | 55.1±0.74 | 57.8±0.69 | 52.9±0.71 | 40.1±0.94* |
| | | $F_2$ | | |
| MALES | | | | |
| Day 4 | 11.0±0.23 | 11.1±0.16 | 9.8±0.16 | 10.2±0.21 |
| Day 14 | 35.5±0.44 | 35.6±0.46 | 33.8±0.39 | 30.6±0.45* |
| Day 21 | 60.2±0.68 | 58.9±0.73 | 56.2±0.70 | 51.5±0.77* |
| FEMALES | | | | |
| Day 4 | 10.6±0.24 | 10.8±0.19 | 9.8±0.18 | 9.7±0.20 |
| Day 14 | 33.9±0.52 | 34.6±0.45 | 32.7±0.51 | 29.1±0.42* |
| Day 21 | 57.4±0.65 | 56.9±0.68 | 54.7±0.77 | 49.0±0.70* |

*Significantly different from control value by Tukey's Test ($P<0.05$).

Table 11. Body and Reproductive Organ Weights at Scheduled Necropsy of $F_0$ and $F_1$ Male Rats Exposed Orally to Sulfur Mustard (Mean ± SE).

| HD | N | Body Weight | Epididymis | | Prostate | | Testes | |
|---|---|---|---|---|---|---|---|---|
| mg/kg/day | | g | g | mg/100g | mg | mg/100g | g | mg/100g |
| | | | | $F_0$ | | | | |
| 0 | 20 | 652.5 ±20.2 | 1.54 ±0.038 | 238 ±6.5 | 605.2 ±30.4 | 94 ±6.7 | 3.70 ±0.069 | 576 ±17.2 |
| 0.03 | 20 | 649.6 ±17.7 | 1.53 ±0.032 | 238 ±6.9 | 700.2 ±34.7 | 109 ±5.3 | 3.72 ±0.070 | 577 ±13.3 |
| 0.1 | 19 | 639.1 ±19.8 | 1.46 ±0.046 | 232 ±8.7 | 604.7 ±30.8 | 95 ±5.1 | 3.79 ±0.093 | 600 ±18.8 |
| 0.4 | 20 | 584.3* ±10.1 | 1.41 ±0.044 | 242 ±7.7 | 587.0 ±36.9 | 101 ±6.3 | 3.62 ±0.146 | 623 ±28.4 |
| | | | | $F_1$ | | | | |
| 0 | 20 | 615.0 ±18.3 | 1.28 ±0.049 | 211 ±11.0 | 887.5 ±39.2 | 147 ±8.3 | 3.50 ±0.158 | 567 ±31.9 |
| 0.03 | 18 | 658.6 ±16.7 | 1.32 ±0.027 | 203 ±6.6 | 805.0 ±60.0 | 124 ±9.4 | 3.65 ±0.087 | 560 ±19.6 |
| 0.1 | 20 | 604.4 ±12.3 | 1.29 ±0.026 | 215 ±5.2 | 900.6 ±43.6 | 149 ±6.4 | 3.79 ±0.067 | 634 ±20.4 |
| 0.4 | 19 | 522.6* ±9.6 | 1.24 ±0.031 | 237 ±6.1 | 749.0 ±38.9 | 144 ±7.4 | 3.45 ±0.097 | 663 ±20.0 |

*Significantly different from control value by Tukey's Test ($P<0.05$), for each pairwise comparsion.

Table 12. Body and Reproductive Organ Weights at Scheduled Necropsy of $F_0$ and $F_1$ Female Rats Exposed Orally to Sulfur Mustard (Mean ± SE).

| HD mg/kg/day | N | Body Weight (g) | Uterus Weight mg | Uterus Weight mg/100g | Ovary Weight mg | Ovary Weight mg/100g |
|---|---|---|---|---|---|---|
| | | | $F_0$ | | | |
| 0 | 20 | 333.6 ±5.3 | 634.5 ±47.9 | 190 ±14 | 160 ±6.4 | 48 ±1.8 |
| 0.03 | 21 | 346.5 ±5.7 | 724.6 ±50.7 | 210 ±15 | 166 ±7.7 | 48 ±2.1 |
| 0.1 | 18 | 339.4 ±5.8 | 721.6 ±49.4 | 215 ±15 | 189 ±10.2 | 56 ±3.0 |
| 0.4 | 20 | 315.6* ±8.3 | 798.5 ±62.5 | 254 ±19 | 156* ±7.3 | 50 ±2.7 |
| | | | $F_1$ | | | |
| 0 | 15 | 354.4 ±10.6 | 652.8 ±66.0 | 184 ±18 | 108 ±4.2 | 31 ±1.2 |
| 0.03 | 20 | 352.8 ±7.1 | 681.6 ±48.0 | 195 ±15 | 111 ±3.2 | 32 ±1.0 |
| 0.1 | 21 | 346.2 ±6.8 | 600.8 ±37.4 | 177 ±14 | 107 ±4.8 | 31 ±1.3 |
| 0.4 | 24 | 312.9* ±8.4 | 624.6 ±41.7 | 205 ±16 | 102 ±4.8 | 33 ±1.3 |

*Significantly diffenent from control value by Tukey's test ($P<0.05$), 657`1`for each pairwise comparison.

A small number of squamous papillomas of the forestomach was also observed in about 10% of the intermediate (8/94) and high dose (10/94) groups (Table 13). These benign neoplasms were composed of a proliferating, nodular to papilliferous exophytic growth of squamous epithelium containing a fibrovascular central core and attached to the underlying epithelium by a stalk of varying thickness. There were relatively few other neoplastic lesions in either the control or treated rats, and none involving the genital tract of either sex was observed. Except for the squamous papillomas of the forestomach, all neoplasms were believed to be spontaneous in nature and not associated with the administration of HD.

Table 13. Pertinent Histomorphologic Lesions

| Dose (Mg/Kg) | Male (FO) 0 | 0.03 | 0.1 | 0.3 | Male (F1) 0 | 0.03 | 0.1 | 0.4 |
|---|---|---|---|---|---|---|---|---|
| Forestomach | | | | | | | | |
| Number Examined | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 |
| Acanthosis, Minimal | 0 | 0 | 1 | 0 | 0 | 2 | 0 | 0 |
| Acanthosis, Mild | 0 | 12 | 5 | 2 | 0 | 15 | 0 | 0 |
| Acanthosis, Moderate | 0 | 0 | 11 | 4 | 0 | 0 | 19 | 4 |
| Acanthosis, Marked | 0 | 0 | 0 | 14 | 0 | 0 | 1 | 16 |
| Inflammation, Subacute, Mild | 0 | 1 | 0 | 3 | 0 | 0 | 0 | 1 |
| Inflammation, Subacute, Marked | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Squamous Papilloma | 0 | 0 | 1 | 2 | 0 | 0 | 2 | 2 |

| Dose (Mg/Kg) | Female (FO) 0 | 0.03 | 0.1 | 0.3 | Female (F1) 0 | 0.3 | 0.1 | 0.4 |
|---|---|---|---|---|---|---|---|---|
| Forestomach | | | | | | | | |
| Number Examined | 27 | 27 | 27 | 27 | 27 | 27 | 27 | 27 |
| Acanthosis, Minimal | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 |
| Acanthosis, Mild | 0 | 20 | 5 | 1 | 0 | 19 | 4 | 0 |
| Acanthosis, Moderate | 0 | 1 | 18 | 3 | 0 | 0 | 20 | 4 |
| Acanthosis, Marked | 0 | 0 | 3 | 23 | 0 | 0 | 2 | 23 |
| Inflammation, Subacute, Mild | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| Squamous Papilloma | 0 | 0 | 3 | 3 | 0 | 0 | 2 | 3 |

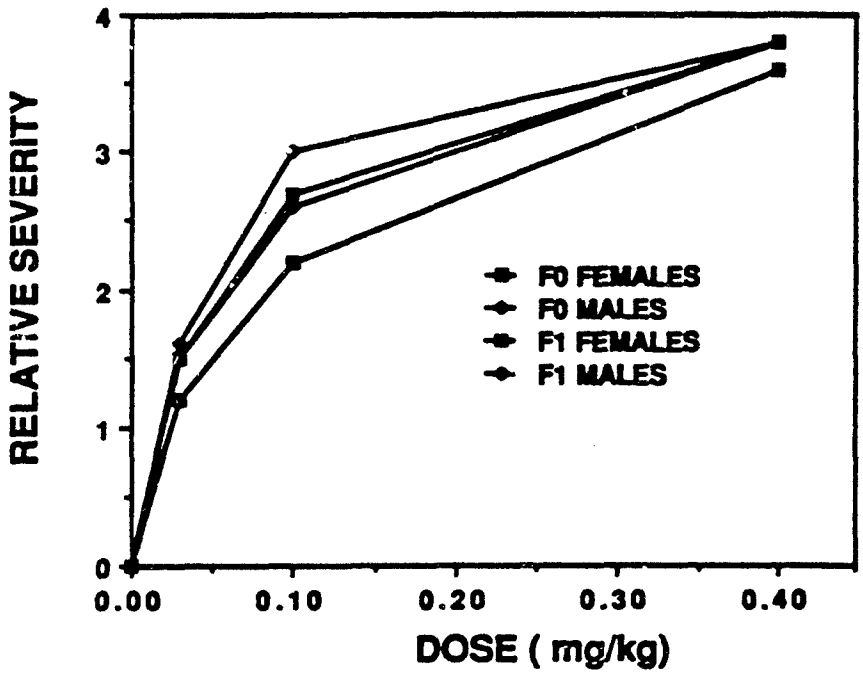


Figure 4. Relative severity of forestomach lesions in male and female adult rats as a function of HD dose.

## DISCUSSION

Results of the present study indicate that exposure, via intragastric intubation, to concentrations of HD between 0.03 and 0.4 mg/kg over two generations did not result in any alterations in the reproductive performance or fertility of the rat. Other than a decrease in growth rate during nursing, no adverse effects to offspring were attributed to HD exposure. Although similar long-term studies have not been conducted, these findings are consistent with previously reported short-term teratology studies of HD. Evidence of teratogenicity was not found in rats exposed up to 0.1 $mg/M^3$ HD via inhalation (McNamara et al., 1975) or 2 mg/kg HD via intragastric intubation (Hackett et el., 1987). The lack of significant effects on reproductive performance, fertility and fetal development in these studies suggests that HD is not a developmental toxic material in the animal model. We have, however, shown HD to be mutagenic in *in vitro* microbial and mammalian mutagenicity test systems using the Ames and CHO/HGPRT assays (Stewart et al., 1989; Jostes et al., 1989). The lack of correlation between the *in vitro* systems and the *in vivo* studies raises some interesting questions regarding HD doses to organs. A major question remaining is whether HD transfer to the body or fetus in its active form is of sufficient magnitude to affect reproduction. Degradation of HD is known to occur in aqueous solutions but studies of HD absorption from the gastrointestinal tract or skin and its subsequent metabolism have not been conducted. No data exist regarding the placental transfer of HD.

Although HD had little effect on reproductive performance and fertility, there were significant signs of maternal toxicity as a result of HD exposure particularly at the high dose. The retarded growth rate of the adult $F_1$ females, the reduced necropsy weights of the $F_0$ and $F_1$ females and the reduced growth rate of the $F_1$ and $F_2$ neonatal pups between day 4 and weaning in both the intermediate and high dose groups are evidence of maternal toxicity. Although food consumption was not measured, it is likely that the lactating females may have been nutritionally stressed because of the lesions in the forestomach to the point of affecting milk production. The mild decrease in body weight is further evidence supporting a decrease in food

intake. No overt signs of anorexia were found and no adverse effect of treatment was evident on physical observations or animal behavior.

Except for a slight reduction in absolute ovary weight of the $F_0$ females at the highest dose, absolute and/or relative male and female reproductive organ weights were unaffected by HD exposure. No evidence of treatment-related effects was found when these organs were pathologically examined.

The forestomach was the primary target organ for HD. Benign neoplasms of the forestomach were found in about 10% of the 0.1 mg/kg and 0.4 mg/kg groups. A dose-related lesion of the squamous epithelium of the forestomach was observed in each generation at all treatment groups. This was the only effect observed at the 0.03 mg/kg dose level although the lesion was mild compared to the other treatment groups. The characteristics of the epithelial lesions were similar to those of the 90-day subchronic study except that in this study pathological changes were observed at lower doses than in the subchronic study. Mild lesions were observed in over 50% of the animals receiving 0.03 mg/kg of HD whereas in the subchronic study lesions were limited to the 0.3 mg/kg group except for one animal receiving 0.1 mg/kg. This difference is probably a result of the longer exposure period of this study (13 vs 22 weeks for females). Dose-related changes in the pathology of reproductive organs were not apparent.

In conclusion, exposure to HD at levels of .03, 0.1 and 0.4 mg/kg/day did not have any adverse effect on reproductive performance or fertility of male or female rats through two consecutive generations. The No-Observable-Effect-Level in this study was $<0.03$ mg/kg for toxicity and $> 0.4$ mg/kg for reproductive effects.

## LITERATURE CITED


Anslow, W.P., D.A. Karnofsky, B.V. Jager, and H.W. Smith. 1948. The intravenous, subcutaneous and cutaneous toxicity of bix (β-chloroethyl)sulfide (mustard gas) and of various derivatives. J. Pharmacol. Exp. Therap. 93: 1-8.

Cassarett, L.J. and J. Doull. 1986. Toxicology. The Basic Science of Poisons, 3rd Ed., MacMillan Publishers, New York, NY.

Dixon, W.J. 1983. BMDP statistical software, University of California Press, Berkley, CA.

Fox, M. and D. Scott. 1980. The genetic toxicology of nitrogen and sulfur mustard. Mutat. Res. 75: 131-168.

Hackett, P.L., R.L. Rommereim, F.G. Burton, R.L. Buscbom and L.B. Sasser. 1987. Teratology studies on lewisite and sulfur mustard agents: Effects of sulfur mustard in rats and rabbits. AD A187495. U.S. Army Medical Research and Development Command, Fort Detrick, Frederick, MD.

Haskin, D. 1948. Some effects of nitrogen mustard on the development of external body form in the fetal rat. Anat. Rec. 102: 493-511.

Jostes, R.F. Jr., R.J. Rausch and L.B. Sasser. 1989. Toxicology studies of Lewisite and sulfur mustard: Genetic toxicity of sulfur mustard (HD) in Chinese hamster ovary cells. U.S. Army Medical Research and Development Command, Fort Detrick, Frederick, MD.

Kramer, C.Y. 1957. Extension of multiple range test to group means with unequal numbers of replications. Biometrics 12: 307-310.

Marshall, E. 1984. Iraq's chemical warfare: Case proved. Science 224: 130-132.

McNamara, B.P., E.J. Owens, M.K. Christensen, F.J. Vocci, D.F. Ford and H. Rozimarek. 1975. Toxicological basis for controlling levels of mustard in the environment. EB-SP-74030. Edgewood Aresenal, Aberdeen Proving Ground, MD.

Rosenblatt, D.H., T.A. Miller, J.C. Dacre, I. Muul, and D.R. Cogley (eds.). 1975. Problem definition of potential environmental pollutants. II. Physical, chemical, toxicological and biological properties of 16 substances. AD A03042[illegible]. In: U.S. Army Medical Bioengineering Research and Development Technical Report 7509. Fort Detrick, Frederick, MD.

Rozmiarek, H., R.L. Capizzi, B. Papirmeister, W.H. Fuhrman, and W.J. Smith. 1973. Mutagenic activity in somatic and germ cells following chronic inhalation of sulphur mustard. Mutat. Res. 21: 13-14.

SAS Institute Inc. SAS User's Guide: Statistics, Version 5 Edition. Cary, N.C.: SAS Institute Inc., 1985.

Sweet, D.V. 1987. Registry of toxic effects of chemical substances Vol 5, p. 1968. U.S. Government Printing Office, Washington, D.C.

Stewart, D.L., E.J. Sass, L.K. Fritz and L.B. Sasser. 1989. Toxicology studies on Lewisite and sulfur mustard agents: Mutagenicity study of sulfur mustard in the *Salmonella* histadine reversion test. U.S. Army Medical Research and Development Command, Fort Detrick, Frederick, MD.

Tukey, J.W. 1953. The problem of multiple comparisons. Ditto, Princeton University, 3996 pp.

Windholz, M. (ed.). 1983. The Merck Index, p. 904, Merck, Rahway, NJ.

Wheeler, G.P. 1962. Studies related to the mechanism of action of cyctotoxic alkylating agents. Cancer Res. 22: 651-688.

Zerbe, G.O. 1979. Randomization analysis of completely randomized design extended to growth and response curves. J. Am. Statistical Assoc. 79: 215-221.

STUDY DATES FOR SULFUR MUSTARD

| | |
|---|---|
| Animals arrival | 10/30/85 |
| Health evaluation | 11/12/85 |
| Begin exposure of $F_0$ generation | 11/26/85 |
| Begin mating $F_0$ generation | 02/24/86 |
| Begin birthing of $F_1$ generation | 03/18/86 |
| Begin necropsy of $F_0$ males and excess females | 04/01/86 |
| Begin necropsy of $F_0$ females | 04/11/86 |
| Wean $F_1$ offspring | 04/09/86 |
| Begin dosing of $F_1$ generation | 07/14/86 |
| Begin birthing of $F_2$ generation | 08/06/86 |
| Begin necropsy of $F_1$ males and excess females | 08/13/86 |
| Begin necropsy of $F_1$ females | 08/29/86 |
| Begin sacrifice of $F_2$ generation | 08/29/86 |

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Data are the property of the U.S. Army and will be archived under the Army's direction in approved facilities.

L B Sasser
L.B. Sasser

11-30-89
Date

## Two-Generation Reproductive Study of Sulfur Mustard (HD) in Rats

### Quality Assurance Statement

Listed below are the phases and/or procedures included in the study described in this report which were reviewed by the Quality Assurance Unit during the period, 10/1/85 to 9/1/86, or specifically for this study, and the dates the reviews were performed and findings reported to management. (All findings were reported to the study director or his designee at the time of the review.)

| Phase/Procedure Reviewed | Review Date | Date Findings Submitted in Writing to Study Director/Management |
|---|---|---|
| Data | 10/07/85* | 10/7/85 |
| Dosing | 12/17/85* | 12/26/85 |
| Data | 12/17/85* | 12/19/85 |
| Data | 12/17/85* | 1/03/86 |
| Vehicle Analysis | 1/17/86* | 1/20/86 |
| Animal Identification | 2/13/86 | 3/03/86 |
| Health Screen | 3/19/86 | 3/10/86 |
| Body Weights | 2/20/86 | 3/10/86 |
| Mating | 2/20/86 | 3/10/86 |
| Dosing | 3/06/86* | 3/10/86 |
| Bleach Analysis | 3/19/86* | 3/20/86 |
| Necropsy | 4/16-18/86* | 4/25/86 |
| Data | 6/04/86* | 6/04/86 |
| Data | 6/2,5/86* | 1/07/87 |
| Data | 6/04/86* | 6/04/86 |
| Mating | 6/20/86 | 7/09/86 |
| Body Weights | 6/20/86 | 7/09/86 |
| Dosing | 6/20/86 | 6/20/86 |
| Necropsy | 7/10/86 | 7/10/86 |
| Vehicle Analysis | 7/11-12/86 | 7/22/86 |
| Dosing | 7/24/86* | 7/31/86 |
| Mating | 7/24/86* | 7/31/86 |
| Body Weights | 7/24/86* | 7/31/86 |
| Dosing | 8/23/86* | 9/08/86 |
| Necropsy | 9/09/86* | 9/09/86 |
| Data | 12/22-23,31/86,1/2,7/87* | 1/07/87 |
| Data | 1/14/88* | 2/01/88 |
| Data | 3/27/89* | 3/28/89 |
| Final Report | 11/6,7 & 12/1/89 | 12/4/89 |

* Reviewed specifically for this study.

Alita M Vowels — Quality Assurance Author — 12/4/89 — Date

R A Gelman — Quality Assurance Author — 12/4/89 — Date

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